



Species identification of staphylococci by amplification and sequencing of the gene compared to the gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

M. Bergeron, O. Dauwalder, M. Gouy, A.-M. Freydiere, M. Bes, H. Meugnier, Y. Benito, J. Etienne, G. Lina, F. Vandenesch, et al.

► To cite this version:

M. Bergeron, O. Dauwalder, M. Gouy, A.-M. Freydiere, M. Bes, et al.. Species identification of staphylococci by amplification and sequencing of the gene compared to the gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *European Journal of Clinical Microbiology and Infectious Diseases*, 2010, pp.343-354. 10.1007/s10096-010-1091-z . hal-00634698

HAL Id: hal-00634698

<https://hal.science/hal-00634698>

Submitted on 22 Oct 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Title

Species identification of staphylococci by amplification and sequencing of the *tuf* gene compared to the *gap* gene and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Running title: *tuf* sequence-based identification of Staphylococci

Marie BERGERON¹, Olivier DAUWALDER^{1,2,3}, Manolo GOUY⁴, Anne-Marie FREYDIERE¹, Michèle BES^{1,2,3}, Hélène MEUGNIER^{1,2,3}, Yvonne BENITO^{1,2,3}, Jerome ETIENNE^{1,2,3}, Gerard LINA^{1,2,3}, François VANDENESCH^{1,2,3*}, Sandrine BOISSET^{1,2,3,*}

¹Hospices Civils de Lyon, Laboratoire de Bactériologie, Centre de Biologie et Pathologie Est, Bron, France

²Université Lyon 1, Centre National de Référence des Staphylocoques, Lyon, France

³INSERM U851, IFR128, Lyon, France

⁴Laboratoire de Biométrie et Biologie Evolutive, CNRS UMR 5558, Université de Lyon, F-69622 Villeurbanne, France

* François Vandenesch & Sandrine Boisset share senior authorship

Corresponding author: mailing address: Laboratoire de Bactériologie, Centre National de Référence des Staphylocoques, 59 Boulevard Pinel, 69677 BRON Cedex, France. Phone: 33 4 72 12 96 64. Fax: 33 4 72 35 73 35. Email: sandrine.boisset@univ-lyon.fr

KEY-WORDS: *Staphylococcus* identification, *tuf* sequencing, *gap* sequencing, phylogeny, MALDI-TOF-MS

Abstract:

Staphylococcal species notably, coagulase-negative staphylococci are frequently misidentified using phenotypic methods.

The partial nucleotide sequences of the *tuf* and *gap* genes were determined in 47 reference strains to assess their suitability, practicability and discriminatory power as target molecules for staphylococcal identification. The partial *tuf* gene sequence was selected and further assessed with a collection of 186 strains including 35 species and sub-species. Then, to evaluate the efficacy of this genotyping method versus the technology of matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS), the 186 strains were identified using MALDI-TOF-MS (Axima® Shimadzu) coupled to the SARAMIS® database (AnagnosTec). The French National Reference Center for staphylococci identification method was used as a reference.

One hundred eighty-four strains (98.9%) were correctly identified by *tuf* gene sequencing. Only one strain was misidentified, and one was unidentified. MALDI-TOF-MS identified properly 138 isolates (74.2%). Four strains were misidentified, 39 were unidentified, 5 were identified at the group (*hominis/warneri*) level and 1 strain was identified at the genus level.

These results confirm the value of MALDI-TOF-MS identification for common species in clinical laboratory practice and the value of the partial *tuf*-gene sequence for the identification of all staphylococcal species as required in a reference laboratory.

Introduction

According to current knowledge, including the newly described species published in 2009-2010, the *Staphylococcus* genus groups together 45 species and 21 subspecies [1, 17, 35, 40, 48]. Staphylococcal species are widely distributed in various environments: the skin and mucous membranes of humans and animals as well as soil, sand, and water. Some staphylococcal species are used as starter cultures for sausage manufacturing in the food industry (*Staphylococcus xylosus* and *S. carnosus*) [7], whereas others are mainly associated with animal diseases such as *S. pseudintermedius* in dogs. Of the 45 species and 21 subspecies, only half have been cultured from human specimens. *S. aureus* is the most clinically relevant staphylococcal species, but coagulase-negative staphylococci (CoNS) are increasingly recognized as etiologic agents of clinical manifestations in humans. CoNS have been identified as a major cause of hospital-acquired infections that typically affect immunocompromised patients with implanted medical devices [52]. Treatment is difficult because many CoNS species carry multiple antibiotic resistances, notably methicillin resistance in approximately 55-75% of nosocomial isolates, as well as glycopeptide resistance, which was initially described in CoNS strains [5, 36]. Identification to the species level is necessary to provide a better understanding of pathogenic potential of various CoNS and could help therapeutic clinical decision [18]. Furthermore, the accurate identification to the species level in reference laboratories is important to establish the role of each staphylococcal species as an infectious agent and to conduct epidemiologic investigations.

Several manual and automated phenotypic identification systems are available, such as the ID32 STAPH[®] strip (bioMérieux), the VITEK 2 GP[®] identification card (bioMérieux) and the PID 61 Phoenix system (Becton Dickinson), but none of these systems are able to accurately identify all staphylococcal species [8, 22, 28]. These methods have been designed mainly for the most frequently encountered species in human clinical samples and are not able to identify rare species and atypical strains such as metabolic variants of common species. More recently, peptide spectra obtained by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) have been used to identify CoNS; this technique has a good performance overall for species encountered in clinical practice [6, 10, 11, 45, 47]. Sample preparation and analysis techniques are simple and can be performed

within minutes. In addition to phenotypic methods, several PCR-sequencing-based methods have been developed for the identification of *Staphylococcus* spp.: the 16S rRNA [4, 15], *hsp60* [13, 26], *sodA* [37], *rpoB* [9, 31], *femA* [51], *tuf* [18, 30] and *gap* [27, 53, 54] genes have been used as targets. Many studies have demonstrated that genotyping methods are superior to phenotypic methods [18, 28]. However, the sequences of some genes are not sufficiently discriminative to differentiate closely related *Staphylococcus* species, and the databases only include a limited number of species. Previous studies suggest that the *tuf* and *gap* genes constitute the most discriminative targets to differentiate closely related *Staphylococcus* species [12]. The *tuf* gene, which encodes the elongation factor (EF-Tu), is involved in peptide chain formation and is a part of the core genome [44]. PCR-based assays targeting the *tuf* gene have been developed for different bacterial genera such as *Enterococcus* [21], *Mycobacterium* [33] and *Staphylococcus* [30]. In the latter case, Martineau *et al.* used hybridization probes (and not DNA sequencing) to differentiate 27 species. The *gap* gene encodes a 42-kDa transferrin-binding protein (Tpn) located within the bacterial cell wall that possesses a glycolytic function, converting D-glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate [34]. Partial sequencing of the *gap* gene has been proposed as an alternative molecular tool for the taxonomic analysis of *Staphylococcus* species [12].

We constructed a *tuf* and *gap* gene sequence database of 47 staphylococcal-type strains and evaluated the performance of this database as a molecular identification tool using a 186-strain collection from the French National Reference Center for staphylococci (CNRSta). Finally, to ascertain the rank of this PCR-sequencing approach among the panel of newly developed techniques, the same collection of strains was also tested using MALDI-TOF-MS technology.

Materials and Methods

Bacterial strains. Type strains representing 47 *Staphylococcus* species and subspecies (Table 1) were used in this study. In addition, 186 strains collected by the CNRSta (Lyon, France) from 1980 to 2008, of both human and animal origin and representing 35 staphylococcal species and subspecies, were included. They were distributed as follows: *S. arlettae* (n = 4), *S. aureus* (n = 9), *S. auricularis* (n = 5), *S. capitis* subsp. *capitis* (n = 5), *S. capitis* subsp. *urealyticus* (n = 6), *S. caprae* (n = 6), *S. carnosus* (n = 3), *S. chromogenes* (n = 4), *S. cohnii* subsp. *cohnii* (n = 5), *S. cohnii* subsp. *urealyticus* (n = 5), *S. epidermidis* (n = 7), *S. equorum* (n = 3), *S. felis* (n = 4), *S. gallinarum* (n = 4), *S. haemolyticus* (n = 8), *S. hominis* subsp. *hominis* (n = 6), *S. hominis* subsp. *novobiosepticus* (n = 4), *S. hyicus* (n = 1), *intermedius* group with *S. delphini* (n = 4), *S. intermedius* (n = 16), *S. pseudintermedius* (n = 4), *S. lentus* (n = 2), *S. lugdunensis* (n = 6), *S. pasteurii* (n = 5), *S. pettenkoferi* (n = 1), *S. piscifermentans* (n = 3), *S. saprophyticus* (n = 7), *S. schleiferi* subsp. *coagulans* (n = 5), *S. schleiferi* subsp. *schleiferi* (n = 8), *S. sciuri* subsp. *carnaticus* (n = 2), *S. sciuri* subsp. *sciuri* (n = 3), *S. sciuri* subsp. *rodentium* (n = 3), *S. simiae* (n = 6), *S. simulans* (n = 6), *S. succinus* (n = 2), *S. warneri* (n = 7), *S. xylosus* (n = 7).

CNR identification. Identification of the above 186 isolates was performed using phenotypic (biochemical characteristics) and genotypic methods. The genus *Staphylococcus* was defined as a Gram-positive cocci with a positive catalase reaction, O/129 compound resistance, bacitracin resistance and nitrofurantoin susceptibility. Coagulase activity on rabbit plasma, heat-stable DNase and the agglutination test (clumping factor, protein A) were used to distinguish *S. aureus* and coagulase-negative Staphylococci (CoNS). In the case of negative coagulase activity or discordant tests, species identification was performed using the ID32 STAPH strip (bioMérieux, Marcy l'Etoile, France). In the case of incorrect identification (unacceptable probability, low confidence factor or no identification), additional tests suggested by the bioMérieux identification system were performed, such as novobiocin susceptibility, oxidase reactions, or the deferroxamine test.

When phenotypic tests were not sufficient for the identification of staphylococcal species, molecular methods were used. Sixty-nine strains required supplementary tests for identification, among which were species not included in the ID32 STAPH database (2006). Fifteen strains were identified by amplification of the 16S-23S

intergenic spacer regions and the restriction enzyme analysis technique as described by Mendoza *et al.* [32]. In addition, other molecular methods were used, such as PCR sequencing of the partial *sodA* gene [37], ribotyping [39] and DNA-DNA hybridization [43]. A PCR based on the amplification of a *S. pasteurii*-specific random amplified polymorphism DNA (RAPD) fragment was performed to identify *S. pasteurii* species [50]. The same technique (amplification of a specific fragment generated by RAPD) was used to identify *S. capitis* ([50] and unpublished). An *agr*-PCR, described by Jarraud *et al.*, permitted the identification of atypical *S. aureus* strains (*i.e.*, lactose negative, mannitol negative, catalase negative or coagulase negative) [20].

Excluding *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticus*, which are discriminated by the ID32 STAPH strip, subspecies were determined using phenotypic or genotypic tests according to the original description of each subspecies: (i) colony pigmentation for *S. capitis* subsp. *capitis* (negative) and *S. capitis* subsp. *urealyticus* (positive) [2], (ii) coagulase activity on rabbit plasma for *S. schleiferi* subsp. *schleiferi* (negative) and *S. schleiferi* subsp. *coagulans* (positive) [19], (iii) novobiocin susceptibility for *S. hominis* subsp. *hominis* (negative) and *S. hominis* subsp. *novobiosepticus* (positive) [24], (iv) nitrate reduction for *S. saprophyticus* subsp. *saprophyticus* (negative) and *S. saprophyticus* subsp. *bovis* (positive) [16] and (v) ribotyping methods for the three subspecies of *S. sciuri* [23, 29].

Bacterial growth and DNA isolation. Chromosomal DNA from all staphylococcal strains were obtained from overnight cultures grown on horse blood trypticase soy agar plates (bioMérieux, Marcy l'Etoile, France) at 37°C. Colonies were suspended in 10 mM/L Tris-HCl buffer, pH 7.0. After centrifugation at 3,450 x *g* for 2 min, the bacterial pellet was resuspended in 100 µL of Tris buffer (10 mM) containing 10 µL of lysostaphin (1 mg/ml) (Sigma, Saint Quentin Fallavier, France), and the mixture was incubated at 37°C for 30 min. DNA purification was completed on the QIAcube apparatus (QIAGEN, Courtaboeuf, France).

Bacterial identification by *tuf* and *gap* sequencing. Based on multiple sequence alignments, a region of the *tuf* gene that is highly conserved among staphylococci was chosen to design the PCR primers. A 660-bp *tuf* DNA fragment was amplified

using the primers stat1 (TTA TCA CGT AAC GTT GGT G) and stat2 (CAT TTC WGT ACC TTC TGG). The PCR program consisted of an initial denaturation step at 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec, and extension at 72°C for 40 sec., and a final extension step for 10 min at 72°C. Gap1-for and Gap2-rev were used to amplify a 931-bp fragment of the *gap* gene as previously described [54]. PCR products were electrophoresed in a 0.8% agarose gel and visualized with SYBR[®] Safe DNA gel stain (Invitrogen) under UV light to confirm the correct size of the amplified product. Amplicons were sequenced using Genoscreen (Institut Pasteur, Lille, France). Both strands were sequenced with stat1 and stat2 oligonucleotides, or with Gap1-for and Gap2-rev for the reference strains. The coding strand was sequenced only for the other 186 strains.

Phylogenetic analysis. Multiple sequence alignments were performed using the ClustalW Program. Phylogenetic trees were generated with the neighbor-joining algorithm [41] applied to synonymous distances (Ks) using the SeaView program [14]. The degree of data support for the tree topology was quantified using the bootstrap method with 500 replications. The *tuf* and *gap* sequences of *Bacillus subtilis* were obtained from GenBank (accession no. NC_000964) and used as the outgroup in the phylogenetic analysis. The identification of the 186 strains of the CNR collection was based on their phylogenetic position and their similarities to the reference strain sequences.

Nucleotide sequence accession numbers. The GenBank accession numbers of the staphylococcal *tuf* and *gap* sequences determined in this study are listed in Table 1.

MALDI-TOF-MS

Staphylococcal strains were sub-cultivated 3 times on Columbia sheep blood agar plates (bioMérieux, Marcy l'Etoile, France) before MALDI-TOF-MS testing. One colony was directly deposited on a MALDI-TOF-MS target plate, and each strain was spotted 4 times. The preparation was overlaid with 1 µl of matrix solution (saturated α-cyano-4-hydroxycinnamic acid). The matrix-sample was crystallized by air-drying at room temperature. Samples were then processed in the MALDI-TOF mass

spectrometer Axima Assurance[®] (Shimadzu, Champs sur Marne, France) using an accelerating voltage of 20 kV in linear mode. The spectra were analyzed in the mass-to-charge ratio (m/z) range of 2 000-20 000. Five hundred laser shots were recorded for each spectrum. Quality controls (*i.e.*, duplicate spots of the *Escherichia coli* CCUG 10979 strain) were performed for each target plate. To identify the strains, the spectra obtained for each isolate were compared to the SARAMIS database for January 2009 (AnagnosTec, Potsdam, Germany). This database includes more than 2 600 SuperSpectra[™], which can be used for automatic microorganism identification, over 35 000 single spectra, and notably 38 *Staphylococcus* species and subspecies. The results of the matching process are expressed as percentages. Values greater than 80% provide reliable identification based on a SuperSpectra[™]. Values between 30 and 80% allow provide identification based on a single spectrum. No identification has been achieved for a score below 30%, as specified by the manufacturer. The SirWeb-MALDI-TOF software (I2A, Perols, France) was used for all experiments to generate the analysis and to export the results to the laboratory informatics system.

Results

***tuf* and *gap* amplification and sequencing.** The utility of amplification–sequencing of the *tuf* and *gap* genes for the identification of staphylococcal species was first determined by analyzing 47 reference strains representing 21 staphylococcal species and 23 subspecies. A partial *tuf* gene sequence (660 bp) was amplified using the primers designed for this study (stat1 and stat2), sequenced and compared. An amplification signal was obtained for all strains tested, and a complete reference database of partial *tuf* gene sequences from the type strains was created for this study. The obtained data were deposited in the GenBank database (accession numbers are presented in Table 1). Similarly, amplification of the partial *gap* gene (931 bp) was performed for the 47 reference strains using the primers described by Yugueros *et al.* [54]. An implemented GenBank database was generated by depositing the 20 missing *gap* sequences that were not deposited by Ghebremedhin [12]. Overall, three species (*S. fleurettii*, *S. vitulinus* and *S. felis*) could not be amplified using the *gap*-specific primers.

***Staphylococcus* phylogeny derived from *tuf* and *gap* sequences.** Multiple alignments of the partial *tuf* and *gap* DNA sequences were carried out using the ClustalX[®] software, and phylogenetic trees were constructed by the neighbor-joining method. Bootstrap support values are indicated at the tree nodes (Figs. 1 and 2). The global topology of the *tuf* tree is in agreement with that constructed by *gap* gene analysis. The two trees revealed three common major clusters (bootstrap values > 90): (i) the “*sciuri* group” (bootstrap value of 97 with *tuf*, 100 with *gap*) including the 3 subspecies of *S. sciuri*, *S. lentus*, *S. vitulinus* and *S. fleurettii*, (ii) the “*intermedius* group” (bootstrap value of 93 with *tuf*, 98 with *gap*) comprising *S. intermedius*, *S. delphini* and *S. pseudintermedius*, and (iii) the “*simulans* group” (bootstrap value of 91 with *tuf*, 100 with *gap*) including *S. simulans*, *S. piscifermentans*, and *S. carnosus*. *S. epidermidis* and *S. saccharolyticus* formed another major cluster in the *gap* tree with a bootstrap value of 95, whereas these two species were not related in the *tuf* tree. In agreement with other methods, the two trees clustered *S. schleiferi*, *S. hyicus*, *S. chromogenes*, *S. muscae*, and *S. lutrae* with the *S. intermedius* group (bootstrap value of 36 with *tuf*, 67 with *gap*), *S. haemolyticus* with *S. lugdunensis* and *S. hominis* and *S. warneri* with *S. pasteurii*, and finally *S. aureus* with *S. simiae*. In both trees, the “*saprophyticus* group” included *S. saprophyticus*, *S. cohnii*, and *S.*

xylosus, with the addition of *S. succinus* and *S. gallinarum* in the *tuf* tree (Figs. 1 and 2). These analyses revealed that the two gene sequences allowed the discrimination of all *Staphylococcus* species, because subspecies of the same species were always clustered together with the exclusion of any other *Staphylococcus* species. Bootstrap values were typically higher for *gap*. Thus, the *gap* gene had a greater discriminatory power than *tuf* for the differentiation of *Staphylococcus* species. However, *tuf* demonstrated greater practicability; a 660-bp amplicon of *tuf* was sufficient for the analysis, versus 900 bp for the *gap* gene. In addition, *tuf* provided a more universal analysis, because it resulted in the amplification of all species, in contrast with *gap* (Table 1). Therefore, the *tuf* gene was selected for further analysis.

Species identification of CNRSta laboratory collection strains by *tuf* sequencing. The *tuf* gene-based identification matched at the species level 184/186 strains obtained from CNRSta (98.9%) (Table 2). Note that for the *S. intermedius* group, identification was considered correct when the *tuf* sequence assigned the identification to the group and not necessarily to the three recently defined species constituting this group: *S. delphini*, *S. pseudintermedius* and *S. intermedius* [42]. For the remaining two strains, one was identified as *S. schleiferi* by CNRSta and as *S. warneri* by *tuf* sequencing, with the latter identification confirmed by *gap* sequencing. The second strain identified as *S. warneri* by CNRSta and confirmed to be *S. warneri* by *gap* sequencing could not be identified by *tuf* sequencing for reasons unknown. Similarly to other molecular methods, *tuf* did not discriminate *Staphylococcus* subspecies, except for *S. cohnii* subsp. *cohnii* and *S. cohnii* subsp. *urealyticus*.

Comparison of MALDI-TOF-MS and *tuf* sequencing for species identification. Forty-four of the 47 reference strains together with the 186 strains from the CNRSta collection were analyzed using the MALDI-TOF technology. The two anaerobic strains (*S. aureus* subsp. *anaerobius* and *S. saccharolyticus*) and *S. fleurettii* were not tested. Five species, *S. kloosii*, *S. muscae*, *S. piscifermentans*, *S. simiae* and two subspecies of *S. succinus* not included in the SARAMIS® database, provided an incorrect (*S. simiae* identified as *S. aureus*) or no identification (Table 3). Concerning the species or subspecies included in the SARAMIS® database, seven reference strains were not identified (*S. auricularis*, *S. caprae*, *S. hyicus*, *S. intermedius*, *S. pasteurii*, *S. pettenkoferi* and *S. schleiferi* subsp. *schleiferi*), whereas for some of

these species, a correct identification was obtained for several isolates from the CNRSta collection (Table 3). For instance, the reference strain of *S. caprae* was not identified, whereas 5 out of 6 isolates from the CNRSta collection were properly identified by MALDI-TOF-MS. The reference strain of *S. warneri* and 5 out of 7 *S. warneri* isolates of the CNRSta collection were identified only at the group *hominis/warneri* level. Overall, 138 out of 186 strains (74.2%) from the CNRSta collection were identified at the species level by MALDI-TOF-MS, and one *S. warneri* strain was identified at the genus level. Four strains were misidentified, and 39 were unidentified. Five *S. warneri* strains were assigned to the group *hominis/warneri*. After exclusion of the CoNS species not included in the database (*i.e.*, *S. simiae*, *S. kloosii*, *S. muscae*, *S. piscifermentans*, and *S. succinus*), the final percentage of correct identifications in the CNRSta collection reached 81.5%.

Discussion

The use of nucleic acid targets provides an alternative technique for the accurate identification of *Staphylococcus* species. Because of the large amount of 16S rDNA sequence data available in public databases, this gene has been the favorite choice in many studies. However due to its lack of discriminatory power, the 16S rDNA sequence is not sufficient for the reliable identification of staphylococcal species [4]. Thus, several targets have been exploited to identify species belonging to the *Staphylococcus* genus. Among these, the *sodA* [37], *rpoB* [9, 31], *hsp60* [25], *dnaJ* [46], *gap* [12, 27] and *tuf* gene [30] sequences have been found to be useful for staphylococcal species identification. Major interest in the use of *tuf* results from the small required size of the amplicon (660 bp) together with the ability to use non-degenerate oligonucleotide primers; these two conditions have not been achieved simultaneously by most other targets. The *tuf* gene has thus emerged as a reliable molecular tool for the accurate identification of *Staphylococcus* species [12, 18, 49]. However, published studies have been limited to the most common staphylococcal species encountered in human diseases [30]. In the present study, we extended the sequence analysis of the *tuf* gene to a total of 47 species and subspecies. Thus, the present study is the most extensive *tuf*-gene sequence-based study to date on staphylococcal species and sub-species.

Considering the phylogeny derived from the *tuf* gene, the global topology of the *tuf* tree, notably the presence of three major clusters, is in agreement with trees constructed based on the analysis of the other genes listed above [9, 12, 26, 30, 37, 46, 49]. The strains belonging to the “*sciuri* group” form an identical cluster in all phylogenetic trees derived from 16S rDNA, *rpoB*, *sodA*, *hsp60*, *dnaJ*, *gap* and *tuf* gene sequences. These strains are all novobiocin resistant and oxidase positive. Similarly, the “*intermedius* group” clusters with *S. schleiferi*, *S. hyicus*, *S. chromogenes*, *S. muscae* and *S. lutrae* by phylogenetic analysis of the 16S rDNA, *rpoB*, *hsp60*, *dnaJ*, *gap* and *tuf* genes. In contrast, the phylogeny obtained using *sodA* is slightly discordant with those phylogenies, because *S. schleiferi*, *S. hyicus*, *S. muscae*, and *S. chromogenes* do not cluster with the “*intermedius* group” using *sodA* [37]. The third major cluster, the “*simulans* group,” is conserved with *sodA*, *rpoB*, *hsp60*, *dnaJ*, *gap* and *tuf* gene analysis but not with 16S rDNA. In addition to these major clusters, the “*saprophyticus* group” appears to be partially conserved in the *tuf* phylogeny (*S. saprophyticus*, *S. cohnii*, *S. xylosus*, *S. gallinarum*) with a low

bootstrap value, in contrast with other gene-derived phylogenies including *S. arlettae*,
S. kloosi, and *S. equorum* in the “*saprophyticus* group” [9, 25, 37] [12, 30, 46, 49].
The other groups with low bootstrap values in the *tuf* phylogeny appeared to be
poorly conserved in the phylogenies derived from other genes.

The present study resulted in the creation of an almost complete reference database
of partial *tuf* gene sequences from type strains. Indeed, the completeness of the
database is essential for reliable identification. Prior to the present work, numerous
species were either not identified or misidentified when relying on the *tuf* GenBank
database; for instance, *S. carnosus* could be misidentified as *S. simulans*, *S.*
gallinarum as *S. saprophyticus*, *S. lentus* as *S. sciuri*, and *S. piscifermentans* as *S.*
simulans. However, both the percentage of similarity (below 97%) and the topology
of the *tuf*-based phylogenetic tree should demonstrate the lack of robustness of such
results.

Considering sub-species identification, *tuf* and *gap* sequencing did not allow
discrimination at the subspecies level except for *S. cohnii* subsp. *cohnii* and *S. cohnii*
subsp. *urealyticus*, as demonstrated for other genes [12, 37]. Thus, molecular
methods are clearly not suitable for identification at the subspecies level, a restriction
with almost no consequences in clinical practice.

Because mass spectrometry is becoming increasingly popular for bacterial
identification, we wondered whether it would outcompete *tuf* sequencing in the
identification of the 47 species and subspecies of staphylococci. It is noteworthy that
no studies have yet explored such a diversity of staphylococcal species. When
comparing the *tuf*-based identification with the MALDI-TOF-MS technology, we
concluded for an overall superiority of the molecular method even though the MALDI-
TOF-MS based method is faster and more cost effective than the molecular method.

As expected the MALDI-TOF-MS with 74.2% of correct identification out-competed
the ID32 STAPH that identified 62.9% of isolates. When excluding species not
included in the databases these percentages were 81.5% versus 75% respectively.

The slight inferiority of the MALDI-TOF-MS versus the *tuf*-based method was rather
unexpected given the number of enthusiastic reports on the performance of this
technology for species identification [6, 10, 11, 45, 47]. Dupont *et al.* analyzed 230
isolates of CoNS representing 20 species. They obtained correct identifications for
93.2% of the isolates using MALDI-TOF-MS, and this percentage reached 97.4%
with exclusion of the species not included in their database [11]. Similarly, Dubois *et*

385 *al.* used the MALDI-TOF-MS Biotyper[®] to identify a collection of 156 strains
386 representing 22 different species and obtained concordant identifications for 99.3%
387 of the species [10]. There are several reasons to explain these apparent
388 discrepancies. First, the SARAMIS database is said to comprise 38 species and
389 subspecies; however, only 15 species or subspecies have a SuperSpectra[®]. It
390 appears that a reliable identification can only be obtained in the latter cases. A similar
391 limitation has been pointed out by Seng *et al.* for the Biotyper database [45]. It is
392 important to note that this drawback has limited consequences in routine clinical
393 practice, because the most frequent species encountered in humans are well
394 represented in both the SARAMIS[®] and Biotyper[®] databases. Hence, correct
395 identification scores as high as 99.3% can be reported for bloodstream isolates in
396 certain studies using MALDI-TOF-MS [47]. Expanding the database to include more
397 species and more strains tested per species would improve the performance of this
398 promising method. Second, the strain collection tested in the present study (the
399 CNRSta collection) not only contains numerous species that are exceptional in
400 clinical practice (but not necessarily never encountered) but also includes isolates of
401 rare species, which were difficult to identify using classical methods and were thus
402 referred to us as a reference laboratory. A third possible reason for the slight
403 inferiority of the MALDI-TOF-MS approach in the present study was that our strain
404 collection contained isolates that had been stored at -20°C for durations ranging from
405 months to several years. This storage period may have altered the phenotypic
406 expression of proteins and thus decreased the performance of the MALDI-TOF-MS
407 approach, which essentially depends on the expression of ribosomal proteins,
408 without affecting the efficiency of the DNA sequencing approach. In conclusion, the
409 *tuf*-based approach appears to be particularly suited for a reference laboratory in
410 which typical and atypical strains of all staphylococcal species are encountered,
411 whereas at present, MALDI-TOF remains more appropriate for routine microbiology
412 practices in clinical laboratories.

414 **Acknowledgments**

415 We thank Christine Gardon, Christine Courtier, Céline Spinelli, and Caroline
416 Bouveyron for technical assistance, Christian Curel, Michel Roch and Nader Baïda
417 (I2A company) for generously providing us with the Shimadzu Axima[®] mass
418 spectrometer, the SARAMIS[®] identification database and the SirWeb MALDI-TOF

419 software.

References

1. Al Masalma M, Raoult D, Roux V (2010) *Staphylococcus massiliensis* sp. nov., isolated from a human brain abscess. *Int J Syst Evol Microbiol* 60:1066-1072
2. Bannerman TL, Kloos WE (1991) *Staphylococcus capitis* subsp. *ureolyticus* subsp. nov. from human skin. *Int J Syst Bacteriol* 41:144-147
3. Bannoehr J, Ben Zakour NL, Waller AS, Guardabassi L, Thoday KL, van den Broek AH, Fitzgerald JR (2007) Population genetic structure of the *Staphylococcus intermedius* group: insights into agr diversification and the emergence of methicillin-resistant strains. *J Bacteriol* 189:8685-8692
4. Becker K, Harmsen D, Mellmann A, Meier C, Schumann P, Peters G, von Eiff C (2004) Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *J Clin Microbiol* 42:4988-4995
5. Biavasco F, Vignaroli C, Varaldo PE (2000) Glycopeptide resistance in coagulase-negative staphylococci. *Eur J Clin Microbiol Infect Dis* 19:403-417
6. Carbonnelle E, Beretti JL, Cottyn S, Quesne G, Berche P, Nassif X, Ferroni A (2007) Rapid identification of *Staphylococci* isolated in clinical microbiology laboratories by matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol* 45:2156-2161
7. Corbiere Morot-Bizot S, Leroy S, Talon R (2007) Monitoring of staphylococcal starters in two French processing plants manufacturing dry fermented sausages. *J Appl Microbiol* 102:238-244
8. Delmas J, Chacornac JP, Robin F, Giammarinaro P, Talon R, Bonnet R (2008) Evaluation of the Vitek 2 system with a variety of *Staphylococcus* species. *J Clin Microbiol* 46:311-313
9. Drancourt M, Raoult D (2002) *rpoB* gene sequence-based identification of *Staphylococcus* species. *J Clin Microbiol* 40:1333-1338
10. Dubois D, Leyssene D, Chacornac JP, Kostrzewa M, Schmit PO, Talon R, Bonnet R, Delmas J (2009) Identification of a variety of *Staphylococcus* species by MALDI-TOF mass spectrometry. *J Clin Microbiol*
11. Dupont C, Sivadon-Tardy V, Bille E, Dauphin B, Beretti JL, Alvarez AS, Degand N, Ferroni A, Rottman M, Herrmann JL, Nassif X, Ronco E, Carbonnelle E (2009) Identification of clinical coagulase-negative staphylococci, isolated in microbiology laboratories, by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and two automated systems. *Clin Microbiol Infect*
12. Ghebremedhin B, Layer F, Konig W, Konig B (2008) Genetic classification and distinguishing of *Staphylococcus* species based on different partial gap, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* gene sequences. *J Clin Microbiol* 46:1019-1025
13. Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW (1996) HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *J Clin Microbiol* 34:818-823
14. Gouy M, Guindon S, Gascuel O (2010) SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 27:221-224

15. Gribaldo S, Cookson B, Saunders N, Marples R, Stanley J (1997) Rapid identification by specific PCR of coagulase-negative staphylococcal species important in hospital infection. *J Med Microbiol* 46:45-53
16. Hajek V, Meugnier H, Bes M, Brun Y, Fiedler F, Chmela Z, Lasne Y, Fleurette J, Freney J (1996) *Staphylococcus saprophyticus* subsp. *bovis* subsp. nov., isolated from bovine nostrils. *Int J Syst Bacteriol* 46:792-796
17. Hauschild T, Stepanovic S, Zakrzewska-Czerwinska J (2010) *Staphylococcus stepanovicii* sp. nov., a novel novobiocin-resistant oxidase-positive staphylococcal species isolated from wild small mammals. *Syst Appl Microbiol* 33:183-187
18. Heikens E, Fleer A, Paauw A, Florijn A, Fluit AC (2005) Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. *J Clin Microbiol* 43:2286-2290
19. Igimi S, Takahashi E, Mitsuoka T (1990) *Staphylococcus schleiferi* subsp. *coagulans* subsp. nov., isolated from the external auditory meatus of dogs with external ear otitis. *Int J Syst Bacteriol* 40:409-411
20. Jarraud S, Mougél C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F (2002) Relationships between *Staphylococcus aureus* genetic background, virulence factors, agr groups (alleles), and human disease. *Infect Immun* 70:631-641
21. Ke D, Picard FJ, Martineau F, Menard C, Roy PH, Ouellette M, Bergeron MG (1999) Development of a PCR assay for rapid detection of enterococci. *J Clin Microbiol* 37:3497-3503
22. Kim M, Heo SR, Choi SH, Kwon H, Park JS, Seong MW, Lee DH, Park KU, Song J, Kim EC (2008) Comparison of the MicroScan, VITEK 2, and Crystal GP with 16S rRNA sequencing and MicroSeq 500 v2.0 analysis for coagulase-negative Staphylococci. *BMC Microbiol* 8:233
23. Kloos WE, Ballard DN, Webster JA, Hubner RJ, Tomasz A, Couto I, Sloan GL, Dehart HP, Fiedler F, Schubert K, de Lencastre H, Sanches IS, Heath HE, Leblanc PA, Ljungh A (1997) Ribotype delineation and description of *Staphylococcus sciuri* subspecies and their potential as reservoirs of methicillin resistance and staphylolytic enzyme genes. *Int J Syst Bacteriol* 47:313-323
24. Kloos WE, George CG, Olgiate JS, Van Pelt L, McKinnon ML, Zimmer BL, Muller E, Weinstein MP, Mirrett S (1998) *Staphylococcus hominis* subsp. *novobiosepticus* subsp. nov., a novel trehalose- and N-acetyl-D-glucosamine-negative, novobiocin- and multiple-antibiotic-resistant subspecies isolated from human blood cultures. *Int J Syst Bacteriol* 48 Pt 3:799-812
25. Kwok AY, Chow AW (2003) Phylogenetic study of *Staphylococcus* and *Micrococcus* species based on partial hsp60 gene sequences. *Int J Syst Evol Microbiol* 53:87-92
26. Kwok AY, Su SC, Reynolds RP, Bay SJ, Av-Gay Y, Dovichi NJ, Chow AW (1999) Species identification and phylogenetic relationships based on partial HSP60 gene sequences within the genus *Staphylococcus*. *Int J Syst Bacteriol* 49 Pt 3:1181-1192
27. Layer F, Ghebremedhin B, König W, König B (2007) Differentiation of *Staphylococcus* spp. by terminal-restriction fragment length polymorphism analysis of glyceraldehyde-3-phosphate dehydrogenase-encoding gene. *J Microbiol Methods* 70:542-549

- 517 28. Layer F, Ghebremedhin B, Moder KA, Konig W, Konig B (2006) Comparative
518 study using various methods for identification of *Staphylococcus* species in
519 clinical specimens. *J Clin Microbiol* 44:2824-2830
- 520 29. Marsou R, Bes M, Boudouma M, Brun Y, Meugnier H, Freney J, Vandenesch
521 F, Etienne J (1999) Distribution of *Staphylococcus sciuri* subspecies among
522 human clinical specimens, and profile of antibiotic resistance. *Res Microbiol*
523 150:531-541
- 524 30. Martineau F, Picard FJ, Ke D, Paradis S, Roy PH, Ouellette M, Bergeron MG
525 (2001) Development of a PCR assay for identification of staphylococci at
526 genus and species levels. *J Clin Microbiol* 39:2541-2547
- 527 31. Mellmann A, Becker K, von Eiff C, Keckevoet U, Schumann P, Harmsen D
528 (2006) Sequencing and staphylococci identification. *Emerg Infect Dis* 12:333-
529 336
- 530 32. Mendoza M, Meugnier H, Bes M, Etienne J, Freney J (1998) Identification of
531 *Staphylococcus* species by 16S-23S rDNA intergenic spacer PCR analysis. *Int*
532 *J Syst Bacteriol* 48 Pt 3:1049-1055
- 533 33. Mignard S, Flandrois JP (2007) Identification of *Mycobacterium* using the EF-
534 Tu encoding (*tuf*) gene and the tmRNA encoding (*ssrA*) gene. *J Med Microbiol*
535 56:1033-1041
- 536 34. Modun BJ, Cockayne A, Finch R, Williams P (1998) The *Staphylococcus*
537 *aureus* and *Staphylococcus epidermidis* transferrin-binding proteins are
538 expressed in vivo during infection. *Microbiology* 144 (Pt 4):1005-1012
- 539 35. Novakova D, Pantucek R, Hubalek Z, Falsen E, Busse HJ, Schumann P,
540 Sedlacek I (2010) *Staphylococcus microti* sp. nov., isolated from the common
541 vole (*Microtus arvalis*). *Int J Syst Evol Microbiol* 60:566-573
- 542 36. Piette A, Verschraegen G (2009) Role of coagulase-negative staphylococci in
543 human disease. *Vet Microbiol* 134:45-54
- 544 37. Poyart C, Quesne G, Boumaila C, Trieu-Cuot P (2001) Rapid and accurate
545 species-level identification of coagulase-negative staphylococci by using the
546 *sodA* gene as a target. *J Clin Microbiol* 39:4296-4301
- 547 38. Probst AJ, Hertel C, Richter L, Wassill L, Ludwig W, Hammes WP (1998)
548 *Staphylococcus condimenti* sp. nov., from soy sauce mash, and
549 *Staphylococcus carnosus* (Schleifer and Fischer 1982) subsp. *utilis* subsp.
550 nov. *Int J Syst Bacteriol* 48 Pt 3:651-658
- 551 39. Regnault B, Grimont F, Grimont PA (1997) Universal ribotyping method using
552 a chemically labelled oligonucleotide probe mixture. *Res Microbiol* 148:649-
553 659
- 554 40. Riesen A, Perreten V (2009) *Staphylococcus rostri* sp. nov., a hemolytic
555 bacterium isolated from the nose of healthy pigs. *Int J Syst Evol Microbiol*
- 556 41. Saitou N, Nei M (1987) The neighbor-joining method: a new method for
557 reconstructing phylogenetic trees. *Mol Biol Evol* 4:406-425
- 558 42. Sasaki T, Kikuchi K, Tanaka Y, Takahashi N, Kamata S, Hiramatsu K (2007)
559 Reclassification of phenotypically identified staphylococcus *intermedius*
560 strains. *J Clin Microbiol* 45:2770-2778
- 561 43. Schleifer KH, Meyer SA, Rupprecht M (1979) Relatedness among coagulase-
562 negative staphylococci: deoxyribonucleic acid reassociation and comparative
563 immunological studies. *Arch Microbiol* 122:93-101
- 564 44. Schmitt E, Guillon JM, Meinel T, Mechulam Y, Dardel F, Blanquet S (1996)
565 Molecular recognition governing the initiation of translation in *Escherichia coli*.
566 A review. *Biochimie* 78:543-554

- 567 45. Seng P, Drancourt M, Gouriet F, La Scola B, Fournier PE, Rolain JM, Raoult
568 D (2009) Ongoing revolution in bacteriology: routine identification of bacteria
569 by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.
570 Clin Infect Dis 49:543-551
- 571 46. Shah MM, Iihara H, Noda M, Song SX, Nhung PH, Ohkusu K, Kawamura Y,
572 Ezaki T (2007) dnaJ gene sequence-based assay for species identification
573 and phylogenetic grouping in the genus Staphylococcus. Int J Syst Evol
574 Microbiol 57:25-30
- 575 47. Spanu T, De Carolis E, Fiori B, Sanguinetti M, D'Inzeo T, Fadda G, Posteraro
576 B (2010) Evaluation of matrix-assisted laser desorption ionization-time-of-flight
577 mass spectrometry in comparison to rpoB gene sequencing for species
578 identification of bloodstream infection staphylococcal isolates. Clin Microbiol
579 Infect
- 580 48. Supre K, De Vlieghe S, Cleenwerck I, Engelbeen K, Van Trappen S, Piepers
581 S, Sampimon OC, Zadoks RN, De Vos P, Haesebrouck F (2010)
582 Staphylococcus devriesei sp. nov., isolated from teat apices and milk of dairy
583 cows. Int J Syst Evol Microbiol
- 584 49. Takahashi T, Satoh I, Kikuchi N (1999) Phylogenetic relationships of 38 taxa
585 of the genus Staphylococcus based on 16S rRNA gene sequence analysis. Int
586 J Syst Bacteriol 49 Pt 2:725-728
- 587 50. Vandenesch F, Perrier-Gros-Claude JD, Bes M, Fuhrmann C, Delorme V,
588 Mouren C, Etienne J (1995) Staphylococcus pasteurii-specific oligonucleotide
589 probes derived from a random amplified DNA fragment. FEMS Microbiol Lett
590 132:147-152
- 591 51. Vannuffel P, Heusterspreute M, Bouyer M, Vandercam B, Philippe M, Gala JL
592 (1999) Molecular characterization of femA from Staphylococcus hominis and
593 Staphylococcus saprophyticus, and femA-based discrimination of
594 staphylococcal species. Res Microbiol 150:129-141
- 595 52. von Eiff C, Peters G, Heilmann C (2002) Pathogenesis of infections due to
596 coagulase-negative staphylococci. Lancet Infect Dis 2:677-685
- 597 53. Yugueros J, Temprano A, Berzal B, Sanchez M, Hernanz C, Luengo JM,
598 Naharro G (2000) Glyceraldehyde-3-phosphate dehydrogenase-encoding
599 gene as a useful taxonomic tool for Staphylococcus spp. J Clin Microbiol
600 38:4351-4355
- 601 54. Yugueros J, Temprano A, Sanchez M, Luengo JM, Naharro G (2001)
602 Identification of Staphylococcus spp. by PCR-restriction fragment length
603 polymorphism of gap gene. J Clin Microbiol 39:3693-3695

Table 1: Sources and gene accession numbers of the bacterial reference strains used in this study

Strain	Source	<i>tuf</i> gene accession number	<i>gap</i> gene accession number
<i>S. aureus</i> subsp. <i>anaerobius</i>	ATCC 35844	HM352930	HM352968
<i>S. aureus</i> subsp. <i>aureus</i>	CCM 885	HM352919	HM352967
<i>S. arlettae</i>	DSM 20672	HM352954	DQ321674 ^a
<i>S. auricularis</i>	ATCC 33753	HM352956	DQ321675 ^a
<i>S. capitis</i> subsp. <i>capitis</i>	CCM 2734	HM352920	DQ321676 ^a
<i>S. capitis</i> subsp. <i>urealyticus</i>	ATCC 49326	HM352921	HM352966
<i>S. caprae</i>	CCM 3573	HM352928	DQ321677 ^a
<i>S. carnosus</i> subsp. <i>carnosus</i>	DSM 20501	HM352953	DQ321678 ^a
<i>S. chromogenes</i>	CCM 3387	HM352952	DQ321680 ^a
<i>S. cohnii</i> subsp. <i>cohnii</i>	CCM 2736	HM352938	DQ321681 ^a
<i>S. cohnii</i> subsp. <i>urealyticus</i>	ATCC 49330	HM352939	HM352971
<i>S. delphini</i>	DSM 20771	HM352940	DQ321682 ^a
<i>S. epidermidis</i>	CCM 2124	HM352922	DQ321683 ^a
<i>S. equorum</i> subsp. <i>equorum</i>	DSM 20674	HM352959	DQ321684 ^a
<i>S. equorum</i> subsp. <i>linens</i>	DSM 15097	HM352965	HM352977
<i>S. felis</i>	ATCC 49168	HM352941	Failed amplification
<i>S. fleurettii</i>	CIP 106114	HM352961	Failed amplification
<i>S. gallinarum</i>	CCM 3572	HM352942	DQ321686 ^a
<i>S. haemolyticus</i>	CCM2737	HM352923	DQ321687 ^a
<i>S. hominis</i> subsp. <i>hominis</i>	DSM 20328	HM352924	DQ321688 ^a
<i>S. hominis</i> subsp. <i>novobiosepticus</i>	ATCC 700236	HM352925	HM352973
<i>S. hyicus</i>	CCM 2368	HM352943	DQ321689 ^a
<i>S. intermedius</i>	CCM 5739	HM352933	DQ321690 ^a
<i>S. kloosii</i>	DSM 20676	HM352951	DQ321691 ^a
<i>S. lentus</i>	ATCC 29070	HM352944	DQ321692 ^a
<i>S. lugdunensis</i>	ATCC 43809	HM352926	DQ321693 ^a
<i>S. lutrae</i>	DSM 10244	HM352945	HM352978
<i>S. muscae</i>	CCM 4175	HM352957	DQ321694 ^a
<i>S. pasteurii</i>	ATCC 51129	HM352929	HM352972
<i>S. pettenkoferi</i>	CIP 107711	HM352963	HM352976
<i>S. piscifermentans</i>	JCM 6057	HM352955	HM352979
<i>S. pseudintermedius</i>	LMG 22219	HM352962	HM352982
<i>S. saccharolyticus</i>	DSM 20359	HM352932	HM352969
<i>S. saprophyticus</i> subsp. <i>bovis</i>	CCM 4410	HM352934	HM352975
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i>	CCM 883	HM352935	DQ321695 ^a
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	ATCC 43808	HM352936	DQ321696 ^a
<i>S. schleiferi</i> subsp. <i>coagulans</i>	JCM 7470	HM352937	HM352980
<i>S. sciuri</i> subsp. <i>carnaticus</i>	ATCC 700058	HM352946	HM352983
<i>S. sciuri</i> subsp. <i>rodentium</i>	ATCC 700061	HM352948	HM352984
<i>S. sciuri</i> subsp. <i>sciuri</i>	ATCC 29062	HM352947	HM352985
<i>S. simiae</i>	CCM 7213	HM352931	HM352970
<i>S. simulans</i>	ATCC 27848	HM352949	DQ321698 ^a
<i>S. succinus</i> subsp. <i>casei</i>	DSM 15096	HM352964	HM352981
<i>S. succinus</i> subsp. <i>succinus</i>	ATCC 700337	HM352958	HM352974
<i>S. vitulinus</i>	ATCC 51145	HM352960	Failed amplification
<i>S. warneri</i>	CCM 2730	HM352927	DQ321699 ^a
<i>S. xylosum</i>	ATCC 29971	HM352950	DQ321700 ^a

ATCC: American Type Culture Collection; CCM: Czech Collection of Microorganisms; DSM = DSMZ: German Collection of Microorganisms and Cell Cultures; JCM: Japan Collection of Microorganisms; LMG = BCCM/LMG = Belgian Coordinated Collections of Microorganisms; CIP: Institute Pasteur Collection

^a Sequences deposited by Ghebremedhin *et al.* [12]

Fig. 1 Phylogenetic tree of the reference strains based on *tuf* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages $\geq 90\%$ are indicated. The tree was rooted using *Bacillus subtilis*.

Fig. 2 Phylogenetic tree of reference strains based on *gap* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages $\geq 90\%$ are indicated. The tree is rooted at its center.

622 Table 2: *Staphylococcus* species and subspecies identified by *tuf* sequencing versus
623 CNRSta

Species and subspecies (number of strains)	CNRSta identification		<i>tuf</i> identification at the species level versus CNRSta identification
	ID32 STAPH (number of correct IDs)	Complementary tests (number of tests performed)	
<i>S. arlettae</i> (n = 4)	1	ITS-PCR (2), <i>sodA</i> (1)	4/4
<i>S. aureus</i> (n = 9)	7	Accuprobe (1) <i>agr</i> PCR (1)	9/9
<i>S. auricularis</i> (n = 5)	5		5/5
<i>S. capitis</i> subsp. <i>capitis</i> (n = 5)	4	Specific PCR (1)	5/5
<i>S. capitis</i> subsp. <i>urealyticus</i> (n = 6)	2	Specific PCR (4)	6/6
<i>S. caprae</i> (n = 6)	4	ITS-PCR (1) DNA-DNA hybridization (1)	6/6
<i>S. carnosus</i> (n = 3)	0	ITS-PCR (1) specific probes (2) [38]	3/3
<i>S. chromogenes</i> (n = 4)	2	Pigmentation (2)	4/4
<i>S. cohnii</i> subsp. <i>cohnii</i> (n = 5)	5		5/5
<i>S. cohnii</i> subsp. <i>urealyticus</i> (n = 5)	5		5/5
<i>S. delphini</i> (n = 5)	0	DNA-DNA hybridization (1) sequencing (4) [3]	5/5 ^a
<i>S. epidermidis</i> (n = 7)	6	ITS-PCR (1)	7/7
<i>S. equorum</i> (n = 3)	1	DNA-DNA hybridization (2)	3/3
<i>S. felis</i> (n = 4)	0	DNA-DNA hybridization (4)	4/4
<i>S. gallinarum</i> (n = 4)	4		4/4
<i>S. haemolyticus</i> (n = 8)	7	ITS-PCR (1)	8/8
<i>S. hominis</i> subsp. <i>hominis</i> (n = 6)	5	β-glucuronidase (1)	6/6
<i>S. hominis</i> subsp. <i>novobiosepticus</i> (n = 4)	0	ITS-PCR (4)	4/4
<i>S. hyicus</i> (n = 1)	1		1/1
<i>S. intermedius</i> group (n = 15)	13	ITS-PCR (2)	15/15
<i>S. lentus</i> (n = 2)	2		2/2
<i>S. lugdunensis</i> (n = 6)	6		6/6
<i>S. pasteurii</i> (n = 5)	0	Specific PCR (5) [50]	5/5
<i>S. pettenkoferi</i> (n = 1)	0	DNA-DNA hybridization (1)	1/1
<i>S. piscifermentans</i> (n = 3)	0	ITS-PCR (1) DNA-DNA hybridization (2)	3/3
<i>S. pseudintermedius</i> (n = 4)	0	Sequencing (4) [3]	4/4 ^a
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i> (n = 5)	5		5/5
<i>S. saprophyticus</i> subsp. <i>bovis</i> (n = 2)	0	DNA-DNA hybridization (2)	2/2
<i>S. schleiferi</i> subsp. <i>schleiferi</i> (n = 9)	8		8/9
<i>S. schleiferi</i> subsp. <i>coagulans</i> (n = 5)	0	ITS-PCR (4), DNA-DNA hybridization (1)	5/5
<i>S. scirui</i> subsp. <i>caraticus</i> (n = 2)	2		2/2
<i>S. sciuri</i> subsp. <i>sciuri</i> (n = 3)	3		3/3
<i>S. sciuri</i> subsp. <i>rodentium</i> (n = 3)	3		3/3
<i>S. simiae</i> (n = 6)	0	DNA-DNA hybridization (6)	6/6
<i>S. simulans</i> (n = 6)	6		6/6
<i>S. succinus</i> (n = 2)	0	<i>sodA</i> PCR (2)	2/2
<i>S. warneri</i> (n = 7)	7		6/7
<i>S. xylosus</i> (n = 7)	7		7/7
	117 (62.9%)	69 (37.1%)	184/186 (98.9%)

624 ^a Assigned to group *intermedius* by *tuf* sequencing

625

626 Table 3. MALDI-TOF-MS identification based on quadruplicate runs of each strain

Species	MALDI identification	
	Reference strains	CNRSta collection strains
<i>S. arlettae</i>	1/1	4/4
<i>S. aureus</i>	1/1	9/9
<i>S. auricularis</i> ^a	0/1	0/5
<i>S. capitis</i> subsp. <i>capitis</i>	1/1	3/5
<i>S. capitis</i> subsp. <i>urealyticus</i>	1/1	3/6
<i>S. caprae</i>	0/1	5/6
<i>S. carnosus</i>	1/1	2/3
<i>S. chromogenes</i>	1/1	3/4
<i>S. cohnii</i> subsp. <i>cohnii</i>	1/1	5/5
<i>S. cohnii</i> subsp. <i>urealyticus</i>	1/1	1/5
<i>S. delphini</i>	1/1	3/4
<i>S. epidermidis</i>	1/1	7/7
<i>S. equorum</i> subsp. <i>equorum</i>	1/1	3/3
<i>S. equorum</i> subsp. <i>linens</i>	1/1	-
<i>S. felis</i>	1/1	2/4
<i>S. gallinarum</i>	1/1	4/4
<i>S. haemolyticus</i>	1/1	8/8
<i>S. hominis</i> subsp. <i>hominis</i>	1/1	6/6
<i>S. hominis</i> subsp. <i>novobiosepticus</i>	1/1	4/4
<i>S. hyicus</i>	0/1	1/1
<i>S. intermedius</i>	0/1	-
<i>S. intermedius</i> group		21/24
<i>S. kloosii</i> ^a	0/1	-
<i>S. lentus</i>	1/1	1/2
<i>S. lugdunensis</i>	1/1	6/6
<i>S. lutrae</i>	1/1	-
<i>S. muscae</i> ^a	0/1	-
<i>S. pasteurii</i>	0/1	3/5
<i>S. pettenkoferi</i>	0/1	0/1
<i>S. piscifermentans</i> ^a	0/1	0/3
<i>S. pseudintermedius</i>	1/1	0/7
<i>S. saprophyticus</i> subsp. <i>bovis</i>	1/1	2/5
<i>S. saprophyticus</i> subsp. <i>saprophyticus</i>	1/1	5/5
<i>S. schleiferi</i> subsp. <i>coagulans</i>	1/1	4/7
<i>S. schleiferi</i> subsp. <i>schleiferi</i>	0/1	4/5
<i>S. sciuri</i> subsp. <i>carinatus</i>	1/1	3/3
<i>S. sciuri</i> subsp. <i>rodentium</i>	1/1	3/3
<i>S. sciuri</i> subsp. <i>sciuri</i>	1/1	2/3
<i>S. simiae</i> ^a	0/1	4/6 ^b
<i>S. simulans</i>	1/1	6/6
<i>S. succinus</i> subsp. <i>casei</i> ^a	0/1	-
<i>S. succinus</i> subsp. <i>succcinus</i> ^a	0/1	0/2
<i>S. vitulinus</i>	1/1	-
<i>S. warneri</i>	1/1 ^c	5/7 ^c
<i>S. xylosus</i>	1/1	4/7

	31/44 (70,5%)	138/186 (74,2%)
627	^a Absent in the SARAMIS database	
628	^b False identification: <i>S. aureus</i> instead of <i>S. simiae</i>	
629	^c Identified as a member of the <i>hominis/warneri</i> group	
630		

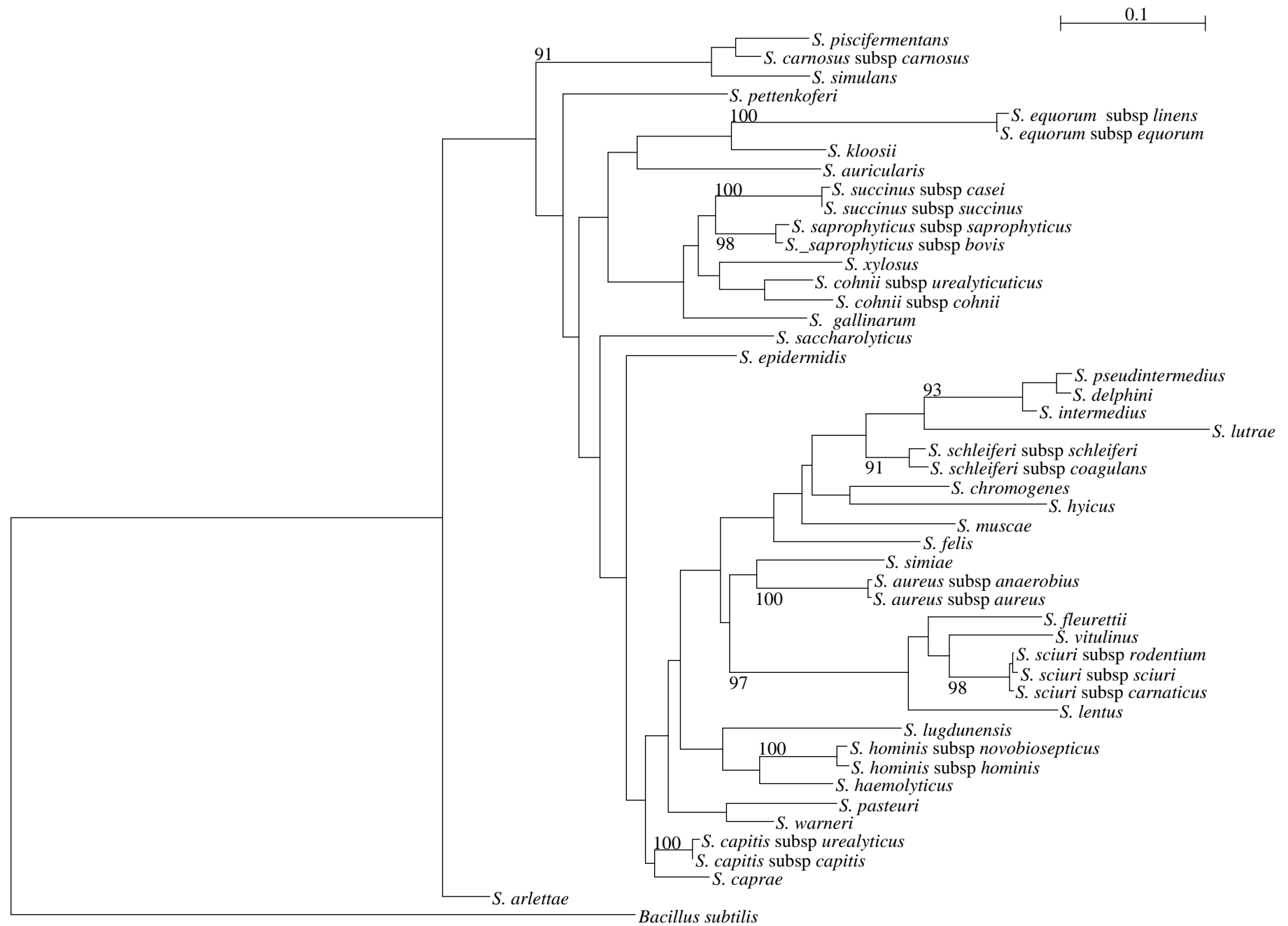


Fig. 1. Phylogenetic tree of the reference strains based on *tuf* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages $\geq 90\%$ are indicated. The tree was rooted using *Bacillus subtilis*.

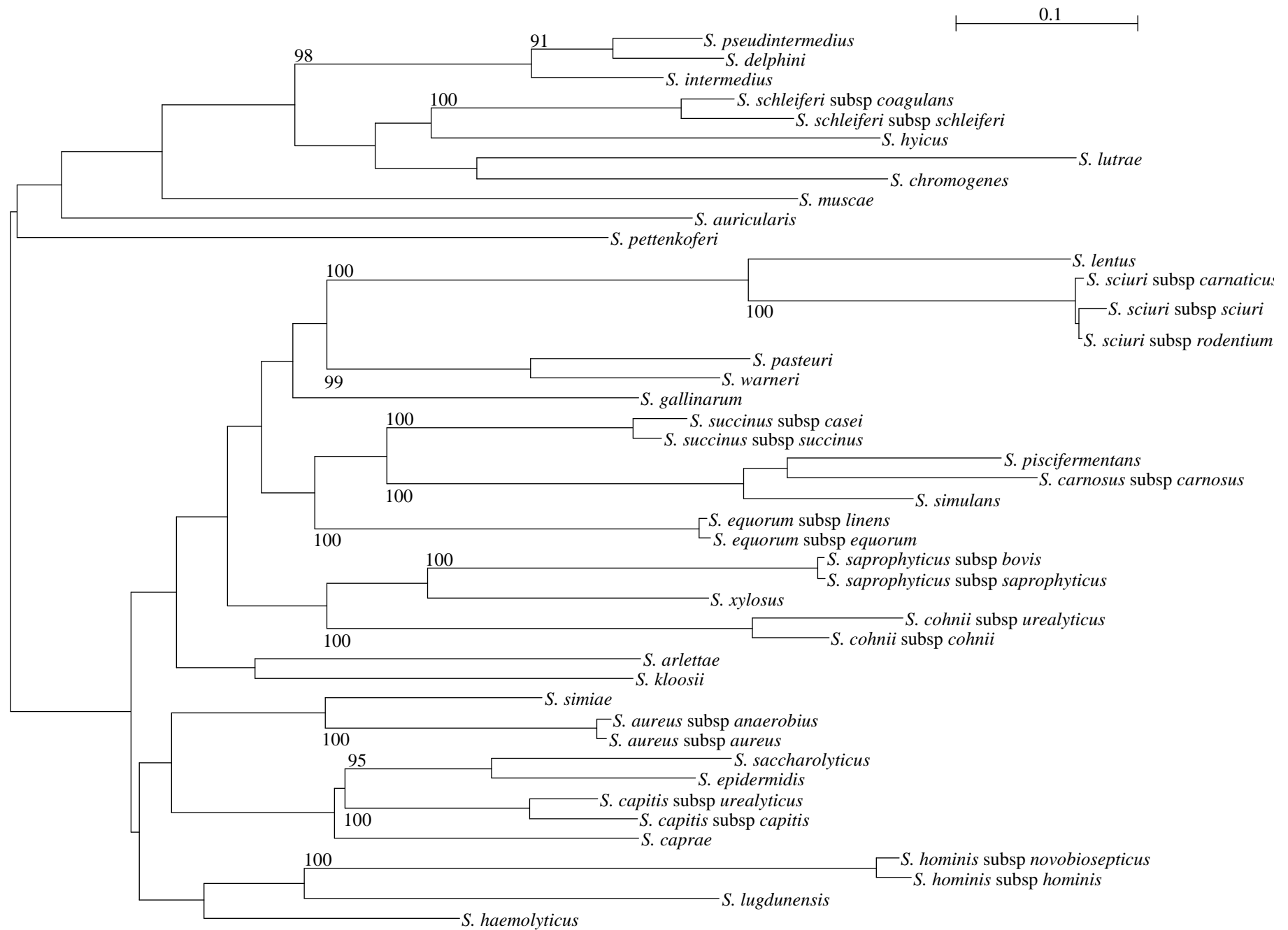


Fig. 2. Phylogenetic tree of reference strains based on *gap* sequences, computed by the neighbor-joining method applied to synonymous distances (Ks). Bootstrap support percentages $\geq 90\%$ are indicated. The tree is rooted at its center.